5. Induction of liver metastasis in rats bearing 7,12-DMBA-induced mammary cancer

Since metastases to vital organs such as the liver and the lungs are common causes of death in breast cancer patients, it is desirable to investigate factors which appear to be relevant to the induction and growth of metastases, and hence to their control. The approach used by the present author and his associates is to study concurrently the primary tumor in the breast and metastatic lesions in the liver and lungs, especially with respect to their biochemical characteristics and their response to hormonal alterations.

It is important to note that while mammary tumors induced by oral administration of chemical carcinogens such as 3-MC and 7,12-DMBA are malignant, they rarely metastasize to distant organs. This phenomenon is as yet unexplained.

a) Method of inducing metastases. Female Sprague-Dawley rats, 50 to 55 days old, were fed a single dose of 20 mg of 7,12-DMBA. Mammary cancers, usually palpable about 60 days after carcinogen feeding, were allowed to grow to a size of 1.0 to 1.5 cm in diameter. A slice of mammary cancer was removed from each tumor-bearing rat under ether anesthesia. Part of the slice was histologically sectioned: the remaining portion placed on a piece of sterile filter paper and weighed on a torsion balance. A tumor-cell suspension was prepared by mincing one part of the tumor (by weight) in 3 parts of tissue-culture medium 199 (by volume). The resulting crude suspension was then filtered through a sterile stainless steel fine-wire mesh (100 wires per inch). No cell counting was done in these experiments.

0.1 ml of the fresh 25 per cent tumor-cell suspension was inoculated into the portal vein of the rat from which the tumor had been removed, as illustrated in a schematic diagram of the rat liver (Fig. 5). The point of entry, which was above the bifurcation of the portal vein (the beginning of the branch to the left lateral lobe), was chosen so that only the median lobe would be affected. Immediately after withdrawal of the needle, a small Gelfoam sponge was placed over the puncture, in order to prevent leaking and contamination of the abdominal cavity.

b) Pathophysiological liver changes in rats receiving tumor-cell suspension. The immediate pathological change seen in the livers of

Schematic diagram of liver of a rat

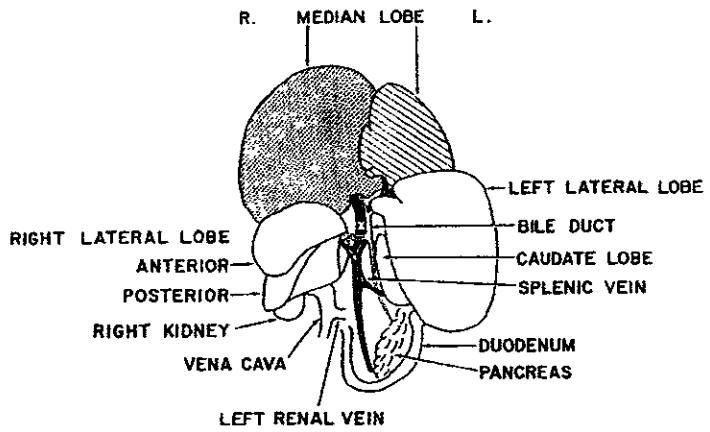

Fig. 5 Schematic diagram of rat liver. X indicates the point of inoculation of a suspension of mammary tumor cells.

rats receiving autologous tumor-cell suspension was massive tumor embolization in the portal veins. On the first day after tumor-cell inoculation, moderate focal to extensive necrosis apparently originating in the periphery of the lobules was observed in the livers. Dilated portal veins containing thrombi, and dilated bile ducts, were clearly visible in the interlobular portal triad. On the fifth day, many intraportal thrombi containing organized tumor acini were seen. Whether these tumor thrombi would grow into metastatic tumors could not be determined at this stage. Morphologically, however, the small tumor foci appeared viable. It is interesting to note that the tumor foci, some clearly degenerating, often formed at the periphery of the thrombus (Fig. 6).

On the 10th day after cell inoculation, many microscopic metastatic tumors were seen. At autopsy, one rat in this group had a grossly visible metastatic tumor. Histologically, these tumors were adenocarcinomas which resembled the original primary cancer (Fig. 7). On the 14th day, more metastatic cancers were visible

Fig. 6 Tumor cells appearing in interlobular portal triad in liver five days after inoculation of mammary tumor-cell suspension. × 100

Fig. 7 Histological appearance of metastatic adenocarcinoma in liver of rat receiving inoculation of suspension of mammary adenocarcinoma cells. × 100

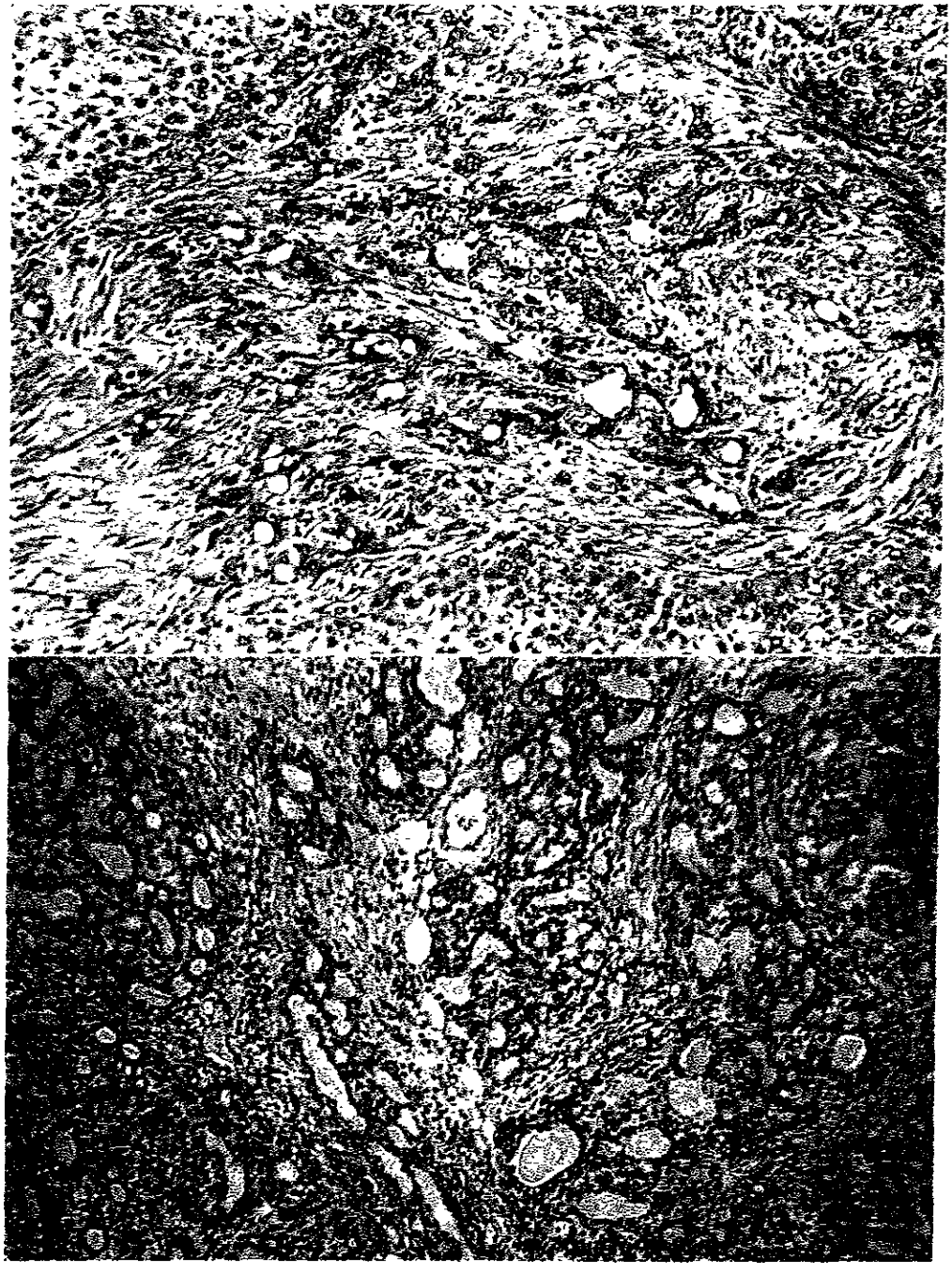

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Table VI

Weight of liver in rats receiving intraportal inoculations of tumor cells

Groups	Days after tumor cell inoculation	Body weight (gm)		Wt. (gm) median lobe (R_1)		Wt. (gm) entire liver (E)		Ratio R_1/E. wt. (%)	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean
Control group		220–289	242±25	1.5–2.8	2.2±0.37	6.5– 9.6	8.3±0.87	6.3–8.6	7.8±0.88
Inoculated group*	1	214–292	256±27	1.8–2.5	2.1±0.24	8.2– 9.8	8.9±0.50	6.8–9.2	8.0±0.95
	5	228–266	245±14	1.1–2.9	1.6±0.37	7.4–10.0	8.2±0.79	4.7–8.3	6.4±1.47
	10	157–277	245±39	0.8–2.2	1.5±0.55	5.1–10.6	8.4±1.77	3.1–9.1	6.1±2.39
	14	178–284	244±32	0.9–2.5	1.5±0.46	5.9– 9.9	8.6±1.45	3.5–8.8	6.1±1.66
	21	179–290	258±36	0.4–2.1	0.8±0.65	5.4– 9.8	8.4±1.44	1.4–7.6	2.9±2.60
	28	224–290	256±18	0.2–2.5	0.9±0.85	6.6–10.0	8.7±1.1	0.7–9.9	3.7±3.41

* Ten rats in each of the inoculated group

grossly. At this stage, small tumor foci had grown into large microscopic tumors. If microscopic metastatic tumor foci were not present, it was assumed that no metastases would develop. It was also at this stage that evidence of liver atrophy was detectable. Intraportal fibrosis was often marked: the affected lobes were small and firm and significantly reduced in weight (Table VI). It was interesting to note that the incidence of microscopic tumors was significantly higher in atrophic livers. The weights of the livers were often normal when metastatic tumors failed to develop.

Between the 21st and 35th days after tumor-cell inoculation, the incidence of microscopic tumors was apparently constant. However, more metastatic tumors became grossly visible on the 35th day. Laparotomy on the 50th day revealed gross evidence of metastatic tumors in the liver in 30% of the rats in this group (Fig. 8).

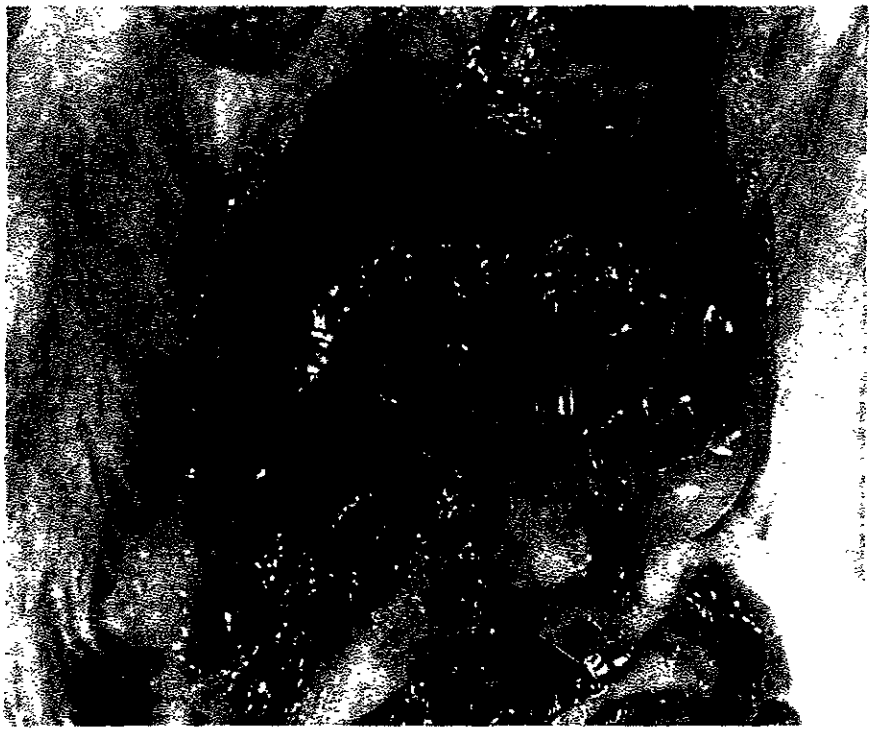

Fig. 8 Gross appearance of metastatic tumors in liver of female rat at autopsy 50 days after receiving mammary tumor-cell suspension.

The current question of interest is why only 30% of the rats receiving autologous tumor cells developed liver metastases. Determination of the conditions which influenced the establishment of metastases is one objective of the present author's future studies.

c) Hormone dependency of induced liver metastases

The hormone dependency of induced liver metastasis in rats bearing 7,12-DMBA-induced mammary cancer has been studied in the present author's laboratory. After confirmation of liver metastases by laparotomy and biopsy, the rats bearing both primary breast cancer and liver metastasis were ovariectomized, the size of each mammary cancer being measured at regular intervals after this procedure. Once regression of the mammary cancer had been established, the abdominal cavities of these rats were re-explored to determine whether the metastasis in the liver had regressed. Preliminary results demonstrated that both the primary mammary cancer and the metastatic liver lesions regressed after excision of the ovaries. Extensive liver metastasis disappeared completely in some of the ovariectomized rats. Residual tumors in the liver were found when the affected lobes were carefully sectioned at the time of necropsy. Histological examination showed atrophy of the metastatic tumor cells (Figs. 9 and 10).

The induction of hormone-dependent metastatic lesions in the liver is highly significant, in that it provides a unique experimental tool for comparative biochemical studies of mammary cancers and their hepatic metastases.

6. *Conclusions*

Mammary cancer can be induced readily in rats of certain strains by a single feeding of 7,12-dimethylbenz(a)anthracene. The induced tumor is unique in that it is hormone-dependent and thus

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Fig. 9 Histological appearance of metastatic mammary adenocarcinoma in liver biopsy from rat 90 days after inoculation of tumor-cell suspension. ×50

Fig. 10 Histological appearance of metastatic mammary adenocarcinoma in liver of same rat, killed four weeks after ovariectomy. Note the atrophy of the metastatic tumor cells. The mammary cancer in this rat also regressed after ovariectomy. ×50

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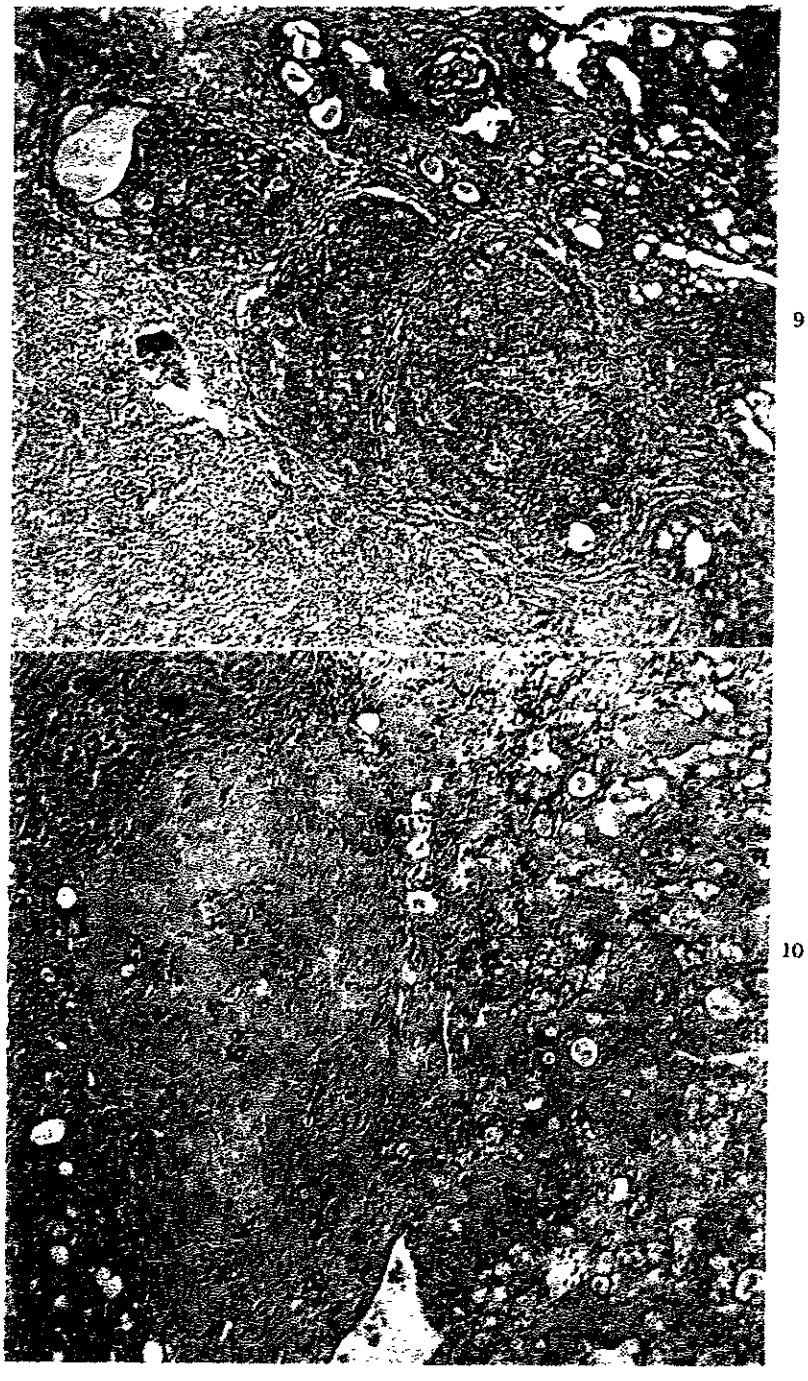

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resembles human mammary cancer. The ease with which mammary cancer can be induced by the technique described, the rapidity of tumor development, and the hormone dependency of the induced tumor make it a valuable experimental model for studies of the etiology, pathogenesis, and control of mammary cancer.

The role of hormones in the induction and maintenance of tumor growth has been discussed in detail on the basis of experiments done in the present author's laboratory and elsewhere. It has generally been agreed that hormones are necessary for the maintenance of tumor growth and hence may be designated as 'co-carcinogens'. However, results obtained in the author's laboratory show that ovarian steroid hormones are probably essential to the process of cancer induction in rat mammary glands.

Whether ovarian hormones exert their action directly upon mammary cells and tumors or indirectly through the pituitary gland is a question which remains to be clarified. It is reasonably clear, however, that prolactin and ovarian hormones (estrogens and progesterone) are both capable of inducing some degree of mammary growth. On the other hand, maximal development of the mammary gland requires both pituitary and ovarian hormones, and interdependence among the pituitary, ovaries, and adrenals plays an integral part in mammary gland growth. Whether or not this same set of relationships also exists in the growth of mammary cancer must yet be determined.

The lack of essential interdependence between mammary function and tumorigenesis was clearly demonstrated by Dao (1962), who showed that enhanced mammary growth is not a prerequisite to mammary tumor development. The concept that the role of hormones in mammary-gland carcinogenesis is to 'stimulate' the glands, thus permitting the development of mammary cancer, is actually not tenable.

The carcinogenic effects of steroid hormones are not yet understood. The steric resemblance between polycyclic hydrocarbons and steroid hormones suggests that the two types of compounds may act at the same sites in biological systems.

IV. Metabolism of Polycyclic Hydrocarbons in the Adrenal and Mammary Glands

1. Toxicity of 3-MC and 7,12-DMBA

The toxic effects of large doses of polycyclic aromatic hydrocarbons have been carefully evaluated. A dosage of 10 mg 3-MC in 1 ml of sesame or olive oil, fed to adult male and female rats daily for 10 or 20 days, caused no body weight decrease in relation to a group fed 1 ml of sesame oil without 3-MC (DAO AND SUNDERLAND, 1959). The rats receiving the carcinogen usually lost their appetite within 3 or 4 days, but regained it a few days later, although they were still receiving the carcinogen. In immature female rats (30 to 35 days old), a dosage of 10 mg of 3-MC daily for 20 days caused 10 to 15% mortality either during the period of carcinogen administration or immediately afterward. Hypophysectomized rats are very susceptible to the toxic effects of the carcinogen. The present author and his associates observed a mortality of 30 to 50% when hypophysectomized rats were fed 10 mg of 3-MC daily for 10 to 20 days. HUGGINS *et al.* (1959) observed no weight loss in rats fed 10 mg of 3-MC daily for as long as 50 days.

A 10-mg dose of 3-MC fed to rats for 10 or 20 days did not cause any decrease in weights of the pituitary, ovaries, and adrenals. It is interesting to note that uterine weights, although unchanged in rats fed 3-MC for 20 days, showed a 100% increase in rats fed 3-MC for only 10 days. Orally administered 3-MC did not suppress the estrous cycle; vaginal smears studied daily during the period when the animals were being fed 3-MC showed that the cycle was still regular. 3-MC-treated rats were mated successfully, and bore normal litters. Decrease in the weights of pituitaries, ovaries, and uteri has also been reported (HUGGINS *et al.*, 1959; SHAY *et al.*, 1959); however, the rats in such experiments were treated with 3-MC repeatedly for more than 50 days. Splenomegaly has also been observed in the present author's laboratory in rats fed 10 mg of 3-MC daily for 20 days.

Greater toxic effects have been observed after a single 20-mg dose of 7,12-DMBA, as follows: diarrhea and weight loss four to five days after carcinogen administration; slower weight recovery; development of leukopenia which lasted ten days or longer before returning to pretreatment level (HUGGINS *et al.*, 1961); gastro-

intestinal tract bleeding (as observed in the present author's laboratory); and, the most interesting development of all, the occurrence of adrenal necrosis and hemorrhage (HUGGINS AND MORI, 1961). Sudden death of the animals, in this case, was attributed to 'adrenal apoplexy'.

(The above-described phenomena will be discussed in detail later in this chapter.)

2. Factors influencing tissue concentration of polycyclic hydrocarbons

a) Factors influencing 3-MC concentration in the mammary glands. Qualitative determinations of the presence or absence of administered polycyclic hydrocarbons in the breast tissue fat, and milk of lactating females of various species have been reported (PEACOCK, 1940; DONIACH *et al.*, 1943; WEIGERT AND MOTTRAM, 1946; LORENZ AND STEWART, 1941; SHAY *et al.*, 1950). Using an Aminco-Bowman spectrophotofluorometer, DAO *et al.* (1959) made quantitative studies of the tissue concentrations of 3-MC after oral feeding of a single dose of the carcinogen. It was found that 24 hours after oral administration of 3-MC to rats, the hydrocarbon was mainly in fatty and breast tissues, levels in other tissues being either very small or insignificant. The milk curd from the stomachs of newborn rats contained a high concentration of 3-MC after a single feeding of 10 mg to the mothers.

The level of 3-MC in breast and fat decreased rapidly within 72 hours, but a significant amount remained in both tissues even at the end of 8 days. The clearance rate of ingested 3-MC was about the same in both fat and breast tissue.

The tissue concentration of 3-MC was proportional to that of the hydrocarbon in the vehicle (BOCK AND DAO, 1961). Thus, higher levels of 3-MC in the mammary glands were obtained after a single feeding of 30 mg of the hydrocarbon than when a 10 mg dose was used. The average values found after 24 hours were 18.0 μg/gm and 6.6 μg/gm respectively. An interesting observation was that there was little difference between the tissue levels of 3-MC in rats fed a single dose and in those fed ten daily doses. Twenty-four hours after the last feeding, the average level in the mammary glands of rats fed one dose of 10 mg was 6.6 μg/gm whereas that in rats fed ten daily doses of 10 mg each was no more than 6.8 μg/gm. When

rats were fed 1.0 ml of sesame oil daily for 9 days prior to a single feeding of 3-MC, there was no significant effect on the uptake of the hydrocarbon by the mammary glands and fat.

These paradoxical results cannot be explained readily. It is certain, however, that they are not attributable to 3-MC cell saturation, since an increase in 3-MC concentration in the vehicle has been shown to be followed by a commensurate increase in the hydrocarbon level in the fat and breast. Could saturation of the cells with the sesame oil vehicle have produced these results? An affirmative answer to this question would be inconsistent with the observation that the uptake of 3-MC by fat and breast was the same in rats fed sesame oil for 9 days prior to 3-MC feeding as it was in animals which had not been 'pretreated'.

BROWN *et al.* (1954) found that feeding rats 3-MC caused an increase in the liver's capacity to demethylate 3-methyl-4-ethyl-aminoazobenzene. Similarly, 3-MC and other hydrocarbons administered orally or by intraperitoneal injection, caused multiple increases in benzopyrene hydroxylase activity in the liver (CONNEY *et al.*, 1957)*. The fact that 3-MC can induce enzymes in the liver for degradative changes in hydrocarbons and aminoazo dyes suggests yet another possibility—that 3-MC itself is metabolized in the same manner.

In studies by BOCK AND DAO (1961), the endocrine state of the rats had little effect on 3-MC concentration, either in the general body fat or in the breast. After a single feeding of 3-MC, the concentration of this substance in the mammary tissues and the clearance rate in normal male and female rats were about the same. The 3-MC levels in both the fat and the mammary glands were lower in pregnant females than in normal nonpregnant females, but higher in either castrated or hypophysectomized animals. These differences, however, could easily be attributed to the differences in the amounts of fat in the mammary glands of rats of differing endocrine conditions. During pregnancy and lactation, the amount of mammary glandular tissue greatly exceeds the amount of fat. After ovariectomy or hypophysectomy, both general body fat and local breast fat are markedly increased.

In the present author's laboratory, experiments were carried out to study the effect of fasting on the incorporation of 7,12-

* DAO *et al.*, 1963: unpublished.

DMBA in the mammary glands and fat. When rats were fasted for seven days or more before the feeding of a single 15 mg dose of 7,12-DMBA, the agent's concentration in the mammary glands and fat was significantly lower than that seen in rats fed a regular diet *ad libitum.* In rats fasted for 7 days, the concentration of 7,12-DMBA 24 hours after a single feeding of 15 mg of the hydrocarbon was 1.1 μg per g of fatty tissue; however, in rats fed a regular diet *ad libitum,* the hydrocabon levels were 12.1 μg per g of mammary tissue and 15.1 μg per g of fatty tissue. On the second day after 7,12-DMBA feeding, no hydrocarbon was detectable in the mammary gland and fat in the fasted rats, whereas a significant amount remained (in these tissues) in the rats fed a regular diet. The loss of body weight resulting from 7 days' fasting ranged from 15 to 44 g, with a mean of 28.8 ± 24.8 g. Additionally, mammary cancer incidence was seen to be definitely lower when rats were fasted more than 5 days prior to the administration of a single 15 mg dose of 7,12-DMBA (Table VII).

The distribution and excretion of radioactivity in rats subsequent to oral feeding of tritium-labeled 3-MC was studied by FLESHER AND SYNDOR (1960). These investigators reported that 60 to 90% of the radioactivity was found in the gastrointestinal con-

Table VII

*Effects of fasting on mammary cancer induction by DMBA**

Groups	No of rats	No. rats with tumors	*No. of rats with tumors and appearance time (days)***					
			60 days	90 days	120 days	150 days	180 days	210 days
Control	15	15 (100%)	1	9	14	15	15	15
2 days****	15	15 (100%)	1	7	10	11	11	15
5 days	14	12 (86%)		6	8	10	11	12
7 days	15	9 (60%)	2	6	8	9	9	9
10 days	15	9 (60%)		2	5	8	9	9

* Single feeding of 15 mg of 7,12-DMBA.

** Time interval between feeding of 7,12-DMBA and appearance of first palpable tumor.

*** Number of days rats were fasted.

tents or feces 24 hours after feeding 10 mg of H^3-3-MC. Less than 2% was recovered in the kidneys, liver, lungs, uterus, brain, muscles, blood, fat, and mammary glands. Of the radioactive materials in the fat and breast tissue, 60 to 70% was toluene-soluble and could be extracted, whereas less than 10% could be extracted from the other tissues. Significant quantities of H^3-3-MC appeared in the urine for 13 to 17 days, and in the feces for 7 to 11 days.

These experiments confirm the earlier studies of DAO, BOCK, AND CROUCH (1959), who showed that an extremely low concentration of the carcinogenic hydrocarbon is sufficient to induce mammary cancer. Assuming that the toluene-extracted radioactivity in FLESHER AND SYNDOR's study was indeed due to 3-MC, their estimated concentrations of 5.8, 6.8, and 5.1 μg per g of mammary tissue from three rats, and 10 and 9.8 μg per g of fat for the first two of the same three rats, respectively, are consistent with the results obtained by DAO, BOCK AND CROUCH (1959).

b) Effects of liver injury on tissue concentrations of polycyclic hydrocarbons. The role of the hepatobiliary system in the intermediary metabolism of polycyclic aromatic hydrocarbons has been studied and established (PEACOCK, 1940; BERENBLUM AND SCHONTAL, 1946; WEIGERT AND MOTTRAM, 1946; HARPER, 1958). Recently FALK *et al.* (1961) showed, by use of C^{14}-benzo(a)pyrene, that the hepatobiliary system is the primary site of metabolism of the administered benzo(a)pyrene. This metabolism consists chiefly of hydroxylation and subsequent conjugation. In the study of carcinogenesis induced by polycyclic aromatic hydrocarbons, it is yet to be determined whether it is the unchanged hydrocarbon or its metabolic products which cause the ultimate intracellular carcinogenic effect. Since the liver plays a major role in the detoxification of mammary carcinogenic hydrocarbons, it should be interesting to determine whether interference with hepatic function will be accompanied by a quantitative increase in the concentration of unmetabolized hydrocarbons in the tissues of selective localization.

In the present author's laboratory, liver injury was induced in rats either by feeding a fatty diet or by intraperitoneal administration of carbon tetrachloride. The vitamin-supplemented fatty diet was composed of 50% lard, 3% casein, 43% glucose, and 4% salt. Rats were fasted for 3 days, and then placed on the fatty diet for 10 days. By the end of 10 days, the liver had undergone diffuse fatty changes.

Grossly, the liver was pale yellow in color, with a glossy appearance caused by excessive fat deposition. Microscopically, the lobules were composed of hepatic cells filled with fat globules. These changes were reversible, and the liver returned to normal shortly after resumption of a regular diet. Intraperitoneal injections of CCl_4 induced necrosis of the liver, most clearly on the second and third days after CCl_4 administration. Recovery was usually seen by the fifth or sixth day.

In the fatty diet experiments, rats were fed a single dose of either 30 mg of 3-MC or 15 mg of 7,12-DMBA on the 11th day. Twenty-four hours later, the breast, body fat, brain, adrenals, lungs, ovaries, heart, kidneys, spleen, uterus, and liver were dissected out for determination of the unmetabolized carcinogen concentrations, according to the method described by DAO *et al.* (1959). In comparison with the previously reported data on carcinogenic

Table VIII

Effect of fatty liver on levels of 3 MC in tissues of rats fed the hydrocarbon*

Tissue	Control (regular diet)	High lipid diet
Fat	19.3**	30.1
Breast	19.3	30.2
Brain	2.4	3.9
Adrenal	8.4	9.2
Lung	0.07***	0.19*
Ovary	6.2	10.2
Heart	0.29	0.54
Kidney	0.06***	0.15*
Spleen	0.16***	0.25*
Uterus	4.6	6.9
Thymus	3.7	4.4
Liver	0.19***	0.09*

* The analysis were made 24 hours after an oral feeding of 30 mg of 3 MC.
** The levels in tissues are Ug/gm of tissues. Each value represents the mean of determination in 5 rats.
*** Values insignificant.

hydrocarbon concentrations in normal mammary glands and many other tissues, the 3-MC level in both breast and body fat were considerably higher in rats fed a high-fat diet. The 3-MC levels in the brain, adrenals, ovaries, and uterus also increased, but the amount of increase was insignificant (Table VIII). It should be pointed out, however, that these results do not necessarily mean that the increased tissue concentration was the result of interference with the metabolism of the administered hydrocarbon in the injured liver. It is conceivable that transportation of the hydrocarbon was greatly facilitated by the presence of excessive fat deposition.

This possibility seems to be supported by experiments with carbon tetrachloride. In these experiments, CCl_4, mixed with mineral oil in a 1:2 proportion, was administered intraperitoneally at a dosage of 0.1 ml per 100 gm of body weight. Central necrosis invariably occurred in all liver lobes within 48 hours after CCl_4 administration. There was rapid regeneration on the fourth day, and the liver appeared entirely normal on the 6th day. In this study, each of the three polycyclic hydrocarbons 7,12-DMBA, BP (benzo-(a)pyrene), and 3-MC was given in 20 mg doses.

Thirty rats were divided into six groups of five animals each, and each of the three polycyclic hydrocarbons was given to 2 groups. Three days before the hydrocarbons were administered, CCl_4 was injected intraperitoneally into one of the two groups in each pair of groups receiving the same hydrocarbon, and the other group in each pair served as controls.

Twenty-four hours after the feeding of carcinogens in all groups, the rats were killed, and the mammary glands, fatty tissues, adrenals, ovaries, and uteri were dissected out. The hydrocarbon concentration in these tissues was estimated by the method previously described (Dao, Bock and Crouch, 1959). The data shown in Table IX indicate that CCl_4-induced injury to the liver has no effect on the concentrations of the unmetabolized hydrocarbons in these tissues.

Kotin *et al.* (1962) demonstrated that CCl_4-induced injury to rat liver interfered with the normal metabolism of benzo(a)pyrene, particularly in relation to the derivatives of 3-hydroxybenzo(a)-pyrene. Clearance of benzo(a)pyrene from the administration sites and from the circulatory system was both delayed and reduced in the injured liver. Bioassay studies of these investigators showed that enhanced tumorigenesis occurred in mice given subcutaneous in-

Table IX

Effect of carbon tetrachloride on levels of different hydrocarbons in tissues of rats fed the hydrocarbon*

Tissues	7,12-DMBA		BP		3-MC	
	CCl_4 treated	Control	CCl_4 treated	Control	CCl_4 treated	Control
Breast	16.2±2.0	22.8	9.3±2.2	12.5	18.4±3.8	22.2
Fat	21.9±2.2	33.0	12.4±3.0	29.5	16.8±3.2	32.7
Adrenal	3.1± .3	3.5	1.1± .2	1.8	1.6± .5	5.3
Ovary	3.3± .3	4.9	0.8± .2	1.6	1.7± .3	3.0
Uterus	0.5± .1	0.7	0.2± .03	0.7	0.3± .1	0.5

* The analysis was made 24 hours after the oral feeding of 20 mg of each of the hydrocarbons.

The levels in tissues are Ug/gm of tissues. Each value represents the mean of determinations in 5 rats.

± Standard deviation.

jections of both CCl_4 and benzo(a)pyrene, as compared with mice receiving benzo(a)pyrene alone.

In experiments in the present author's laboratory, CCl_4 liver injury has not caused any increase in the concentration of the administered hydrocarbon in the lipophilic sites. The relationship between liver injury and hydrocarbon-induced mammary carcinogenesis is being studied in this laboratory.

3. Selective induction of adrenal necrosis by 7,12-DMBA

a) 7,12-DMBA-induced adrenal necrosis. In studies of 7,12-DMBA-induced mammary carcinogenesis in rats, HUGGINS AND MORII (1961) first reported the observation of hemorrhage and necrosis of the adrenal cortex in animals receiving a single feeding of 20 mg of 7,12-DMBA. The damage to the adrenal cortex was limited to the two inner zones, the *zona glomerulosa* and *medulla* remaining intact.

The morphological changes occurring in the damaged adrenal cortex were most pronounced on the third day after a single feeding

or intravenous injection of 20 or 30 mg of 7,12-DMBA, the adrenal gland appearing dark red and markedly swollen, with edematous periadrenal tissues. Microscopically, the *zona fasciculata* and the *zona reticularis* were completely destroyed. Massive hemorrhage and extensive karyolysis were seen throughout the inner two zones: however, the *zona glomerulosa* and the *medulla* were unaffected.

When a single dose of 30 mg of 7,12-DMBA was given, adrenal necrosis and hemorrhage occurred in 100% of the rats so treated, about 50% of these rats dying 2 or 3 later days, apparently from adrenal apoplexy. Regeneration of the damaged tissue in the adrenal cortex eventually was seen in the survivors. One of the characteristic changes noted during the regenerative period was extensive calcification in a horseshoe-shaped area at the corticomedullary junction. This microscopic appearance persisted as long as six or seven months after onset of the initial injury.

b) Selectivity of 7,12-DMBA. Studies with other carcinogenic hydrocarbons revealed that induction of selective adrenal necrosis is not a property possessed in common by all carcinogenic hydrocarbons (HUGGINS AND MORII, 1961). Among the most powerful cancer-inducing agents are 3-MC, Benzo(a)pyrene, 4-dimethylaminostilbene, and 2-acetylaminofluorene; none of these induced adrenal hemorrhage and necrosis. Compounds such as 9,10-dimethylanthracene (9,10-DMA), benz(a)anthracene, 7-methylbenz(a)anthracene, and 1,4-dimethylphenanthrene (1,4-DMP), while obviously containing major portions of the structure of 7,12-DMBA, fail to induce any adrenocortical damage. Apparently the full complement of 4 rings, as seen in 7,12-DMBA, is necessary in order to induce damage to the adrenal cortex; 9,10-DMA and 1,4-DMP, for example, have the same structure as 7,12-DMBA except that 9,10-DMA lacks a ring on its *a* face, and 1,4-DMP lacks a ring on its *b* face.

In discussing the relationship of 7,12-DMBA's molecular structure to adrenal necrosis, HUGGINS AND MORII (1961) pointed out that the hydrogen atoms in the molecule are not of major importance in this respect, since replacement of the hydrogen atoms in 7,12-DMBA with deuterium atoms does not abolish the corticolytic effect. Noting that the structures of 7,12-DMBA and hydrocortisone are sterically similar, and that hydrocortisone is synthesized in the inner two zones of the cortex, these investigators postulate that it is the structural resemblance between the two

compounds which permits 7,12-DMBA to enter the cortex and to accommodate itself to the molecular sites where hydroxycorticosteroids are synthesized. 7,12-DNBA, an electron donor, forms a charge-transfer complex with an electron acceptor. Once 7,12-DMBA is localized in a closely fitting site, charge transfer sets off a chain of events ultimately resulting in the death of adrenocortical cells. Since most polycyclic aromatic carcinogenic hydrocarbons react by forming strong charge-transfer complexes with appropriate electron acceptors (SZENT-GYORGYI *et al.*, 1960), the most important factor in producing adrenocortical damage, according to HUGGINS AND MORII, must be the steric geometry, which is a unique property of 7,12-DMBA.

Our question now may be posed as follows: If the structural resemblance between hydroxycorticosteroids and 7,12-DMBA is of primary importance, what is the exact mechanism by which 7,12-DMBA exerts its action in producing damage to adrenocortical cells? Obviously, such a question cannot be answered directly at present. However, indirect evidence suggests that adrenocortical secretory activity may be closely related to the induction of adrenal necrosis by 7,12-DMBA.

HUGGINS (1961) showed that adrenal necrosis induced by 7,12-DMBA partially could be prevented if the adrenal glands were rendered inactive by hypophysectomy. (The adrenal glands in hypophysectomized rats became atrophic and thus had little or no secretory activity). Administration of A.C.T.H. restored the susceptibility of the adrenal cortex to damage by 7,12-DMBA in hypophysectomized rats (MORII AND HUGGINS, 1962). These investigators demonstrated that adrenal necrosis did not occur in immature female rats after a single feeding or intravenous injection of 7,12-DMBA, despite a high concentration of the hydrocarbon in the adrenal glands of such rats. Nevertheless, the corticosterone content of the adrenal glands was considerably lower in these animals than in mature rats: in fact, the corticosterone content in the adrenal glands rose progressively as age increased. These findings again suggest that secretory activity is correlated with the 7,12-DMBA-induced damage to the adrenal cortex. Since no charge-transfer complex was formed by 7,12-DMBA with corticosterone, the biological electron acceptor must be assumed to have been part of some biological mechanism concerned with the synthesis of the adrenal corticosteroid hormones.

4. Inhibition of 7,12-DMBA-induced adrenal necrosis by other polycyclic hydrocarbons

a) Effects of polycyclic hydrocarbons on the corticosterone content of the adrenal glands. Studies of the effects of 3-MC and 7,12-DMBA on adrenal corticosterone synthesis revealed that both 3-MC (administered in single or repeated doses) and 7,12-DMBA (given in a single feeding) caused a significant decrease in the corticosterone content of the adrenals (Dao *et al.*, 1963). Corticosterone synthesis began to diminish on the second day after hydrocarbon administration, and was most pronounced on the third or fourth day. The fact that plasma and adrenal corticosterone levels similarly decreased suggests that synthesis of adrenocortical hormones in general was reduced. Whereas 7,12-DMBA produced necrosis of the adrenal cortex, 3-MC did not cause any structural damage of adrenocortical cells. Since both 3-MC and 7,12-DMBA were effective in suppressing the synthesis of corticosterone in the adrenals, the question arises as to whether the inhibitory effect was due to the same mechanism in both instances. To answer this question, the corticosterone content of the adrenals was determined in rats receiving a non-necrotizing dose of 7,12-DMBA.

Although a preliminary experiment revealed that a single feeding of 10 mg of 7,12-DMBA in 1 ml of sesame oil failed to induce a significant depletion in the corticosterone content of rat adrenals, additional experiments must be carried out before concluding that the mechanism by which 3-MC inhibits the synthesis of adrenal corticosterone is different from that by which 7,12-DMBA inhibits it (Dao *et al.*, 1963). Recently, Huggins *et al.* (1963) reported that intravenously injected 0.5–5 mg doses of 7,12-DMBA caused a significant decrease in the corticosterone content of rat adrenals. In Huggins' experiments, adrenal apoplexy was observed in rats receiving as little as 3 mg of 7,12-DMBA intravenously. In the present author's laboratory, adrenal apoplexy was not observed in rats receiving as much as 10 mg of 7,12-DMBA by oral feeding. Whether this discrepancy is attributable to the difference between the routes of administration must yet be determined.

The ascorbic acid contents of adrenal glands and plasma in normal and carcinogen-treated rats were also determined (Dao *et al.*, 1963). In 3-MC-treated rats, there was a continuous rise in adrenal ascorbic acid from the first day to the fifth after a single

feeding of the carcinogen. The level then returned to normal and remained so to the end of the experimental period. In 7,12-DMBA-treated rats, a rapid decline in adrenal ascorbic acid began on the second day after carcinogen feeding, the low level to which it dropped was maintained until the end of the experimental period. The plasma ascorbic acid of 3-MC- or 7,12-DMBA-treated rats was unchanged, indicating that the synthesis of ascorbic acid in other sites was not affected.

The mechanism by which 3-MC stimulates ascorbic acid synthesis remains a mystery (BURNS AND SHORE, 1961; BOYLAND AND JONDORF, 1962). Although the relationship between ascorbic acid and adrenal steroid synthesis is not yet understood, the observation of HAYANO *et al.* (1956), that ascorbic acid may inhibit 11β-hydroxylation in beef adrenals, leads the present author to assume that the stimulatory effect of 3-MC on adrenal ascorbic acid synthesis may be associated with the inhibition of corticosterone synthesis in the adrenal cortex. Whether 3-MC suppresses corticosterone synthesis by inhibition of 11β-hydroxylation is now under study.

b) Antinecrotic effect of metopirone. HUGGIN's postulation—that the structural similarity between hydrocorticosteroids and 7,12-DMBA is of primary importance in 7,12-DMBA-induction of adrenal damage—influenced CURRIE *et al.* (1962) to believe that attrition of the functional activity of the adrenal cortex might influence the incidence of 7,12-DMBA-induced adrenal necrosis. 2-Methyl-1,2-di(3-pyridyl)-1-propanone (metopirone, metyrapone, or SU 4885), an amphenone analogue, is known to inhibit 11β-hydroxylation, hence to interfere with corticosterone and hydrocortisone syntheses (CHART *et al.*, 1958). Metopirone, injected subcutaneously every 3 hours for 36 hours, successfully inhibited the induction of adrenal necrosis by 7,12-DMBA which was given 24 hours after the first dose of metopirone (CURRIE *et al.*, 1962).

DAO AND TANAKA (1963) confirmed CURRIE's observation, and also demonstrated that 10 mg of metopirone, injected intraperitoneally every 4 hours for 3 injections, protected the adrenal cortex from injury by 7,12-DMBA given 4 hours after the last dose of metopirone. When 7,12-DMBA dosage was increased from 20 to 30mg, the number of rats protected by the same dose of metopirone was reduced. Although the exact mechanism by which metopirone inhibits the induction of adrenal necrosis is not clearly understood,

the synthetic capacity of the adrenal gland apparently governs the effect of 7,12-DMBA on the cortical cells which synthesize adrenocortical hormones.

1,1-Dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane (o,p'-dichlorodiphenyldichloroethane, o,p'-DDD), another amphenone analogue, is known to induce adrenal necrosis in dogs, and consequently to decrease the synthesis of adrenocortical hormones (VILAR AND TULLNER, 1959). It is not known, however, whether a non-necrotizing dose of o,p'-DDD would inhibit adrenocortical hormone synthesis. In experiments in the present author's laboratory (DAO AND TANAKA, 1963), o,p'-DDD, given at optimal doses prior to the administration of a necrotizing dose of 7,12-DMBA failed to protect the adrenal cortex from damage by 7,12-DMBA. This result cannot be readily explained. (o,p'-DDD was given orally in the earlier experiments [VILAR AND TULLNER, 1959], but intraperitoneally in the more recent experiments [DAO AND TANAKA, 1963]. It is doubtful, however, that the difference in the administration route would make a radical difference in the effect of o,p'-DDD.)

c) Antinecrotic effects of polycyclic hydrocarbons. The inhibitory effect of metopirone on both adrenal corticosteroid synthesis and 7,12-DMBA-induced adrenal necrosis led to the possibility that 3-MC, which was also capable of suppressing corticosterone synthesis, might likewise inhibit 7,12-DMBA-induced necrosis. DAO AND TANAKA (1963) discovered that 3-MC, given to rats prior to 7,12-DMBA feeding, could indeed protect the adrenal cortex against 7,12-DMBA-induced necrosis. A single dose of 30 mg of 3-MC fed to rats 48 hours prior to the feeding of 20 or 30 mg of 7,12-DMBA completely protected the adrenal cortex against injury caused by 7,12-DMBA. This remarkable observation led to the postulation that the mechanisms by which metopirone and 3-MC inhibited 7,12-DMBA-induced adrenal necrosis were probably similar.

In the discussion of the mechanism by which various polycylic hydrocarbons inhibit 7,12-DMBA-induced adrenal necrosis, it is necessary to mention another possibility. It has been well established since the early work of CONNEY, MILLER AND MILLER (1956) that administration of a small dose of a polycyclic hydrocarbon to rats induces a marked increase in hydroxylating enzymes in the liver. More recently it has been shown that the gastrointestinal tract, lungs and kidneys are also sites where hydroxylating enzymes increase after injection of

polycyclic hydrocarbons (WATTENBERG, LEONG AND STRAND, 1962; GELBOIN AND BLACKBURN, 1964). It is conceivable that markedly increased concentrations of hydroxylating enzymes in the liver and other sites following pretreatment with polycyclic hydrocarbons make possible the metabolic destruction of 7,12-DMBA before it can reach the adrenal cortex in sufficient quantity to inflict damage upon the gland. To test the validity of such a hypothesis, benzpyrene hydroxylase in the liver, gastrointestinal tract and adrenal glands of rats receiving polycyclic aromatic hydrocarbons has now been extensively studied in our laboratory.

Since other polycyclic hydrocarbons such as benzo(a)pyrene, benz(a)anthracene, phenanthrene, and anthracene, have proved effective as inhibitors of 7,12-DMBA-induced adrenal necrosis (DAO AND TANAKA, 1963), it may thus be seen that 3-MC is not unique in respect to this interesting phenomenon. Of even greater interest, however, is the observation that 7,12-DMBA-induced adrenal necrosis is effectively blocked by a non-necrotizing dose of 7,12-DMBA.

For the hydrocarbons tested, the minimal doses insuring 100% protection of the adrenal cortex against 7,12-DMBA-induced necrosis are as follows: 3-MC, 5 mg; 7,12-DMBA, 10 mg; benzo-(a)pyrene, 10 mg; benz(a)anthracene, 10 mg; anthracene, 25 mg; and phenanthrene, 100 mg. Obviously, the most effective protector is 3-MC, whereas phenanthrene is barely active. It is interesting to note that the antinecrotic potencies of these compounds more or less seem to parallel their carcinogenic activities. Thus, the minimum effective antinecrotic doses of 3-MC, 7,12-DMBA, and benzo(a)pyrene are closely comparable, whereas the minimum antinecrotic dose of the noncarcinogen, phenanthrene is about 20 times that of 3-MC.

It should be noted that these data show a similarity to the findings obtained in a study of the effect of hydrocarbons on the sebaceous glands (BOCK AND MUND, 1958). In that study, the degree to which polycyclic aromatic hydrocarbons destroyed sebaceous glands was parallel to the carcinogenic potency of the compounds.

HUGGINS *et al.* (1963) confirmed the observations made by DAO AND TANAKA (1963), and also tested several other aromatic amines, all of which proved effective in protecting the adrenal cortex against 7,12-DMBA-induced injury. In HUGGINS' study, the hydrocarbons were administered by intravenous injection, which leads to the conclusion that the hydrocarbons need not pass through the gastrointestinal tract in order to exert their protective effect.

d) Significance of inhibition of 7,12-DMBA-induced adrenal necrosis. Why is the adrenal cortex so severely and so selectively damaged by 7,12-DMBA? The fact that this property is unique strongly supports the assumption that specificity of the 7,12-DMBA's molecular structure is of the utmost importance. At present, that specificity appears to be due to steric resemblance between 7,12-DMBA and the adrenocortical steroids.

Since 7,12-DMBA inflicts damage to the adrenal gland only in the two inner zones of the cortex, where corticosterone and hydrocortisone are synthesized, the question arises: Why does not 3-MC induce adrenal necrosis? If structural similarity is the primary factor governing the entry of 7,12-DMBA into the cortical cells, as HUGGINS postulates, does the failure of 3-MC to induce adrenal necrosis indicate that 3-MC cannot enter the cortical cells? If 3-MC cannot enter the cortical cells, how can it inhibit 7,12-DMBA-induced adrenal damage? Furthermore, does 3-MC inhibit the synthesis of adrenocortical steroids—possibly indirectly, by inhibiting anterior pituitary function and thus interfering with the secretion of ACTH? These questions must yet be answered.

Studies by HUGGINS and by the present author and his associates have provided overwhelming evidence that 7,12-DMBA-induced damage to the adrenal cortex bears some relationship to the adrenal gland's capacity to synthesize steroids. The biosynthesis of adrenocortical steroids from cholesterol involves a number of oxidative processes carried out by various oxidative and hydroxylating enzymes in the adrenal cortex. During these oxidative processes, electron transfers take place between steroids and a number of acceptor sites. If a polycyclic hydrocarbon is present, it will attach itself to these acceptor sites as do steroid hormones.

7,12-DMBA-increased adrenal necrosis can be inhibited by the administration of any of various polycyclic hydrocarbons, including 7,12-DMBA itself, prior to the necrosis-inducing dose of 7,12-DMBA. Assuming that 7,12-DMBA combines in different ways at necrotizing and non-necrotizing concentrations, could this inhibition conceivably be due to successful retention of binding sites by previously bound hydrocarbons when a necrotizing dose of 7,12-DMBA competes for those sites? An understanding of the mechanism by which polycyclic hydrocarbons inhibit 7,12-DMBA-induced adrenal damage may lead to a better understanding of the mechanism by which they induce cancer in rat mammary glands.

5. Conclusions

The concentration and clearance of polycyclic hydrocarbons in the fat, mammary glands, and other tissues of rats have been extensively studied in the present author's laboratory. Chemical carcinogenesis in the mammary glands of the rat seems to involve two factors associated with the administered carcinogen; first, the concentration of the carcinogen in the target tissue; and second, the persistence of the carcinogen in that tissue. The adipose tissue of the mammary glands apparently functions as a storage depot for the carcinogen. The amount of 3-MC in the target tissue is only 5 to 6 μg per g of tissue after a single feeding of 30 mg; hence it seems that only a trace amount of the carcinogen is required for induction of mammary cancer.

In the studies performed by Bock and Dao, the rats' endocrine state seemed to be of relatively small importance in respect to hydrocarbon concentration in the target tissues. Liver injury induced by carbon tetrachloride did not seem to affect the concentration of unchanged hydrocarbons in the mammary glands. Fasting the rats prior to feeding a carcinogenic hydrocarbon appeared to lower the uptake of the hydrocarbon considerably, and to enhance the clearance of the hydrocarbon from the target tissues. It is of interest that tumor incidence is lowered and the latent period prolonged in fasted rats receiving a maximal dose of carcinogenic hydrocarbon.

The vehicle evidently was important in relation to the transport of an orally administered hydrocarbon. An aqueous suspension of 3-MC fed by mouth failed to induce mammary cancer in rats, even when large doses were given (Dao *et al.*, 1960).

A unique property of 7,12-DMBA proved to be its capacity to induce massive necrosis and hemorrhage in the inner two zones of the adrenal cortex. The majority of rats recovered, despite the adrenal damage, when only 20 mg of 7,12-DMBA was given. When 30 mg of 7,12-DMBA was given, however, 30 to 50% of the rats died of adrenal necrosis and hemorrhage within three days, and the rest succumbed later.

The mechanism by which 7,12-DMBA induces adrenal necrosis is unknown. It has been postulated that two factors are important: the resemblance between the molecular structures of 7,12-DMBA and the hydroxycorticosteroids, and the formation of a chargetransfer complex between the powerful electron donor, 7,12-DMBA, and an appropriate electron acceptor. This hypothesis must be explored further.

It is highly significant that the adrenal gland's ability to synthesize corticosteroids is paralleled by its susceptibility to 7,12-DMBA-induced necrosis. The suppression of corticosterone synthesis apparently protects the adrenal cortex against damage caused by 7,12-DMBA. 3-MC has been found to decrease the synthesis of corticosterone. Administered 48 hours prior to the feeding of 7,12-DMBA, 3-MC completely inhibits the necrosis inducing effect of the 7,12-DMBA. This remarkable protective phenomenon is not unique to 3-MC, but is shared by many other hydrocarbons, including even 7,12-DMBA itself, in non-necrotizing doses. These phenomena are of utmost importance, since they provide a new means for studying the mechanism of hydrocarbon-induced carcinogenesis.

V. Summary

Induction of mammary cancers in rats by means of hormones and chemical carcinogens has been described. Whereas hormones from the anterior pituitary have little or no direct effect on mammary cancer induction, their presence seems to be important for the induction of mammary cancer by ovarian hormones. Interdependent effects of the pituitary, ovaries, and adrenals seem to be essential for mammary gland development and mammary tumorigenesis. It has been shown, however, that functional development of the mammary glands is not a prerequisite to cancer induction.

Mammary carcinogenesis induced by chemical carcinogens offers a unique experimental model for study of the etiology, pathogenesis, and control of mammary cancer. The roles of hormones in mammary carcinogenesis induced by polycyclic hydrocarbons have been defined. It is the present author's view that ovarian hormones have dual functions, both inductive and supportive, in the development of mammary cancer.

The fate and clearance of hydrocarbons after administration have been discussed. The data suggest that hydrocarbon concentration in the target tissues and the slow rate of clearance are important factors in the carcinogenic process.

The selective induction of both adrenal necrosis and breast cancer by polycyclic aromatic hydrocarbons are remarkable phenomena. Hypotheses attempting to explain the selective induction of adrenal necrosis by 7,12-DMBA have been presented in this chapter. Especially significant is the finding that the induction

of adrenal necrosis by 7,12-DMBA is closely associated with the synthesis of corticosterone in the adrenal cortex. Suppression of corticosterone synthesis inhibits 7,12-DMBA-induced adrenal necrosis. Among the agents capable of suppressing corticosterone synthesis are metopirone and many polycyclic hydrocarbons. The capability of polycyclic hydrocarbons to protect the adrenal cortex against damage from 7,12-DMBA is an extremely important discovery in the study of the mechanism of carcinogenesis. Further elucidation of this phenomenon will provide a major advance toward total understanding of the mechanism of carcinogenesis induced by polycyclic hydrocarbons.

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